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# Matrix Metalloproteinase 9 and Vascular Endothelial Growth Factor Are Essential for Osteoclast Recruitment into Developing Long Bones

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**Abstract.** Bone development requires the recruitment of osteoclast precursors from surrounding mesenchyme, thereby allowing the key events of bone growth such as marrow cavity formation, capillary invasion, and matrix remodeling. We demonstrate that mice deficient in gelatinase B/matrix metalloproteinase (MMP)-9 exhibit a delay in osteoclast recruitment. Histological analysis and specialized invasion and bone resorption models show that MMP-9 is specifically required for the invasion of osteoclasts and endothelial cells into the discontinuously mineralized hypertrophic cartilage that fills the core of the diaphysis. However, MMPs other than MMP-9 are required for the passage of the cells through unmineralized type I collagen of the nascent bone collar, and play a role in resorption of mineralized matrix. MMP-9 stimulates the solubilization of unmineralized cartilage by MMP-13, a collagenase highly ex-

pressed in hypertrophic cartilage before osteoclast invasion. Hypertrophic cartilage also expresses vascular endothelial growth factor (VEGF), which binds to extracellular matrix and is made bioavailable by MMP-9 (Bergers, G., R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, and D. Hanahan. 2000. *Nat. Cell Biol.* 2:737–744). We show that VEGF is a chemoattractant for osteoclasts. Moreover, invasion of osteoclasts into the hypertrophic cartilage requires VEGF because it is inhibited by blocking VEGF function. These observations identify specific actions of MMP-9 and VEGF that are critical for early bone development.

**Key words:** matrix metalloproteinase • VEGF • osteoclast recruitment • endothelial cell • bone development

## Introduction

Bone resorption is essential for the development and maintenance of the skeleton. The main cell responsible for this process is the osteoclast. In embryonic long bones, osteoclasts appear in bone at an early developmental stage (Scheven et al., 1986; Blavier and Delaissé, 1995). First, mononucleated osteoclast precursors enter the mesenchyme surrounding the bone rudiments, proliferate, differentiate into tartrate-resistant acid phosphatase (TRAP)<sup>1</sup>-positive cells, and migrate together with endothelial cells through the nascent bone collar. Then, they invade the calcified cartilage, filling the core of the diaphysis while fusing and differentiating into mature osteoclasts, and transform the core of the bone into a marrow cavity.

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<sup>1</sup>Abbreviations used in this paper: M-CSF, macrophage colony-stimulation factor; MMP, matrix metalloproteinase; TRAP, tartrate-resistant acid phosphatase; VEGF, vascular endothelial growth factor.

The molecular mechanisms directing osteoclastic resorptive activity to specific areas of the bone have generated much interest. Osteoclasts are generated from hematopoietic stem cells under the control of several systemic and local factors (Hofbauer et al., 2000). Osteoclast differentiation factor (Kong et al., 1999), osteoprotegerin (Simonet et al., 1997), macrophage colony-stimulating factor (M-CSF) (Felix et al., 1990; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990), vascular endothelial growth factor (VEGF) (Niida et al., 1999), interleukin-1 (Jimi et al., 1999), and tumor necrosis factor- $\alpha$  (Kobayashi et al., 2000) are involved in osteoclast differentiation. TGF- $\beta$  (Dieudonné et al., 1991; Zheng et al., 1994), M-CSF (Fuller et al., 1993), hepatocyte growth factor (Fuller et al., 1995a), interleukin-8 and macrophage inflammatory protein-1 $\alpha$  (Fuller et al., 1995b), C3 component of complement (Mangham et al., 1993), osteopontin (Faccio et al., 1998; Terai et al., 1999), and bone sialoprotein (Raynal et al., 1996) help target the resorptive activity of osteo-

clasts to specific sites of the matrix. In addition, proteinases of the matrix metalloproteinase (MMP) family are indispensable for the recruitment of osteoclasts in developing metatarsals (Blavier and Delaissé, 1995), and this role is distinct from synergy with cysteine proteinases in solubilizing calcified matrix in the resorption zone (Everts et al., 1998; Saftig et al., 1998). The specific MMP(s) responsible for these osteoclastic activities, and its (their) mode of action have not been determined (Delaissé et al., 2000).

MMP-9 is a candidate for regulating these functions because it is highly expressed in osteoclasts (for review see Vu and Werb, 1998). In embryonic bone, before the formation of the marrow cavity, MMP-9 is highly expressed in cells in the mesenchyme surrounding bone rudiments; as the marrow cavity develops, MMP-9 expression is detected in osteoclasts in the core of the diaphysis (Reponen et al., 1994; Blavier and Delaissé, 1995; Jemtland et al., 1998). Subsequently, the marrow cavity expands longitudinally and at either end interacts with the epiphyseal area to form the growth plates and the metaphysis, areas that are very critical for bone growth and (re)modelling. MMP-9 is then expressed in typical (TRAP<sup>+</sup>) osteoclasts localized along the mineralized longitudinal septae of the metaphysis, as well as in osteoclast-like cells that are localized next to the nonmineralized transverse septae that separate them from the last row of hypertrophic chondrocytes (Jemtland et al., 1998; Vu et al., 1998). This is exactly at the invasion front of the growth plates, where endothelial cells are also abundant. Because these cells are localized against cartilage and differ from osteoclasts to some extent, they are sometimes called chondroclasts. Importantly, the histology of MMP-9 null mice shows that it is a key proteinase for the invasion of the growth plate, but does not support a role of MMP-9 in the actual solubilization of mineralized matrix by osteoclasts (Vu et al., 1998). In addition, a study combining *in situ* zymography, MMP-9 antagonists, and immunohistochemistry showed that MMP-9 acts on the nonmineralized transverse septae of the growth plate (Lee et al., 1999). It is not clear whether MMP-9 exerts a simple mechanical role, such as solubilizing the transversal septae, or plays a more subtle role, such as generating signals that render the cells invasive. Indeed, there are strong indications that MMP-9 exerts its role in invasion of the growth plate through the release of molecules sequestered in the extracellular matrix (Vu et al., 1998). VEGF is one such molecule, since blocking its function leads to impaired invasion of the growth plate, mimicking the phenotype of MMP-9-negative bones (Gerber et al., 1999).

In this study, we analyzed metatarsals of MMP-9-negative and MMP-9-positive embryos well before the formation of the growth plate, and found that the recruitment of the very first osteoclasts in these bones is affected by lack of MMP-9. By using a combination of histological analysis, bone resorption models, invasion chamber experiments, and MMP and VEGF antagonists, we show that the role of MMP-9 in this process is clearly distinct from the role other MMPs play in other steps of bone development. MMP-9 specifically regulates proteolysis of nonmineralized cartilage and release of ECM-bound VEGF, exerting direct chemotactic activity on osteoclasts.

## Materials and Methods

### Mice and Reagents

All animal experiments were performed according to approved protocols following institutional guidelines at the Center for Clinical and Basic Research, the Finsen Laboratory, and the University of California at San Francisco. Mouse embryos for the bone resorption models were littermates from MMP-9<sup>+/-</sup> parents, backcrossed to C57B1/6J for two generations. Mouse embryos for the histomorphometry study were littermates from MMP-9<sup>+/-</sup> × MMP-9<sup>-/-</sup> parents. The mating times were within a 4-h period. The phenotype of each mouse was determined by gelatin zymography of calvarial extracts (Eeckhout et al., 1986) and their genotype by PCR analysis of tail DNA. The wild-type gelatinase B allele was detected by PCR with wild-type primers wt-1 (5'-GCA TAC TTG TAC CGC TAT GG-3') and wt-2 (5'-TAA CCG AGG TGC AAA CTG G-3'). These primers generate a 300-bp fragment. The targeted gelatinase B allele was detected using two primers complementary to the neomycin resistance gene, Neo-1 (5'-ATG ATT GAA CAA GAT GGA TTG CAC G-3') and Neo-2 (5'-TTC GTC CAG ATC ATC CTG ATC GAC-3'). Together these primers generate a 480 bp-fragment. Investigations of MMP-9 expression during development (*in situ* hybridization, immunohistochemistry, and zymography) were done in NMRI strain mice.

Rabbit anti-human MMP-9 antibody (Nielsen et al., 1997) was a gift from Dr. B.S. Nielsen and Dr. N. Borregaard (Finsen Laboratory and Rigshospitalet, Copenhagen). Rat anti-mouse CD34 antibody (Garlanda et al., 1997) was a gift from Dr. C. Garlanda (Pharmacology Institute Mario Negri, Milan, Italy). Recombinant human MMP-13 was a gift from Dr. C. Lopez-Otin (University of Oviedo, Oviedo, Spain). MMP-9 was purified to homogeneity from cultures of BHK cells transfected with mouse MMP-9 and provided by Dr. M. Ferreras (Center for Clinical and Basic Research). Recombinant human VEGF and recombinant human Flt-1/Fc chimera were provided by R&D Systems. Recombinant mouse mFlt-IgG chimera was a gift from Dr. Napoleone Ferrara (Genentech, Inc., San Francisco, CA). GM6001, a general MMP inhibitor, was from AMS Scientific. The other reagents were from suppliers described previously (Delaissé et al., 1985; Blavier and Delaissé, 1995; Sato et al., 1998).

### Analysis of MMPs by Zymography

Calvariae and tibiae were extracted as described (Eeckhout et al., 1986) in a buffer containing 10 mM cacodylate-HCl, pH 6, 1 M NaCl, 0.1 mg/ml Triton X-100, 0.2 mg/ml Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 10<sup>-6</sup> M ZnCl<sub>2</sub>. The two metatarsal triads of each embryo were extracted together in 500 µl extraction buffer for 24 h, and extracts were concentrated four to five times, before analysis by zymography (Lefebvre et al., 1991). To determine whether there were compensatory changes in other proteolytic activities in the absence of MMP-9, a variety of zymography conditions were used: gelatin and casein as substrate, 7.5–12% acrylamide concentrations, and incubation times of 18–60 h.

### Bone Resorption Models

Bone resorption was evaluated with well established models (Delaissé et al., 1985; Blavier and Delaissé, 1995), on littermates of heterozygote parents. Timed pregnant mothers were injected at day 16 (day of vaginal plug discovery is designated day 0) with 100 µCi <sup>45</sup>Ca. The middle three metatarsals and the tibiae were isolated at day 17, and calvariae were isolated at day 18. The middle three metatarsals were kept together as triads. The bone explants were placed on filter paper floating on 400 µl culture medium in 24-well plates. The medium consisted of BGJb solution supplemented with ascorbate (50 µg/ml), glutamax (500 µg/ml), and albumax (1 mg/ml). In addition, the media for long bones were supplemented with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) (Blavier and Delaissé, 1995), and those for calvariae with parathyroid hormones (2 × 10<sup>-8</sup> M) (Delaissé et al., 1985). Cultures lasted usually for 4 d. We measured the <sup>45</sup>Ca released every day in the media and that left in the tissue at the end of the culture. The demineralization of the bone explants during the cultures was then expressed cumulatively as percentage of total amount of radioactivity. The release of <sup>45</sup>Ca from paired experimental groups was compared by the analysis of variance for multiple comparisons.

### Histomorphometry

Metatarsal triads (consisting of the middle three metatarsals) were obtained from littermates of MMP-9<sup>+/-</sup> × MMP-9<sup>-/-</sup> parents. Histological

procedures, the histochemical method for TRAP staining, and the determination of numbers and distributions of TRAP<sup>+</sup> cells and of their nuclei in longitudinal sections of the diaphysis were performed as described previously (Blavier and Delaissé, 1995). These analyses were performed by an investigator unaware of the genotype. Cells and their nuclei were scored as "in" upon their invasion into the calcified cartilage filling the core of the diaphysis, thus indicating their ability to invade. For each metatarsal triad, countings were typically performed in five sections at 20  $\mu$ m from each other and the mean of these five counts was calculated. The results were expressed as mean number of cells or nuclei in one section of metatarsal triad. Counts in +/+ and -/- bones were compared by using the analysis of variance. The analysis of cell and nucleus counts has always led to the same conclusions concerning invasion; however, for clarity, the illustrations in this work show only counts of nuclei because they are more representative of a process where new mononucleated cells are generated and progressively undergo fusion to multinucleated cells.

### Immunohistochemistry

Paraffin sections were treated with 0.45% H<sub>2</sub>O<sub>2</sub> in ethanol to inactivate endogenous peroxidase. For detection of CD34, the sections were digested with trypsin, blocked with casein, incubated overnight with a 1:100 dilution of anti-CD34 antibody, and treated successively with a 1:20 dilution of biotinylated anti-rat antibody and peroxidase-conjugated extravidin. For detection of MMP-9 (Nielsen et al., 1997), the sections were treated three times for 5 min in 10 mM Tris/1 mM EDTA, pH 9, in a microwave oven, blocked with BSA and 0.5% Triton, incubated overnight with a 1:400 dilution of anti-MMP-9 antibody, and finally reacted for 30 min with Dako EnVision<sup>TM</sup> (Peroxidase, Rabbit, K4003). The CD34 and MMP-9 immunoreactivity was visualized by developing the slides in DAB, and H<sub>2</sub>O<sub>2</sub> control MMP-9 immunostainings performed on MMP-9-deficient bones were negative. When CD34 immunostaining and TRAP activity staining were performed on the same sections, immunostainings were done first, followed by TRAP histochemistry. Sections were counterstained with Erlich's hematoxylin.

### In Situ Hybridization

In situ hybridizations were performed on paraffin sections as reported (Graber et al., 1995). A fragment of the 3' end of mouse MMP-9 cDNA overlapping the stop codon (1633–1913) was subcloned into CR11-TOPO cloning vector (Invitrogen), and antisense and sense digoxigenin-labeled riboprobes (of 281 bp) were prepared with SP6 and T7 by using an RNA labeling kit (Boehringer). The hybridized probe was detected by using alkaline phosphatase-conjugated antidigoxigenin antibody and NBT-BCIP substrate mix (Boehringer). The sections were counterstained with methylene blue. For localization of VEGF expression, <sup>35</sup>S-labeled antisense probes were prepared and used as described previously (Gerber et al., 1999).

### Invasion and Chemotaxis Assay

For invasion experiments with MMP-9-positive and MMP-9-negative osteoclasts, osteoclasts were generated from marrow of MMP-9<sup>+/+</sup> and MMP-9<sup>-/-</sup> mice, cultured in the presence of M-CSF and osteoclast differentiation factor (Lacey et al., 1998). For VEGF chemotaxis experiments, osteoclasts were generated from marrow of NMRI mice, cocultured with primary osteoblasts in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Akatsu et al., 1992). They were detached by sequential treatment with 0.1% bacterial collagenase (wt/vol) plus 0.1% dispase (wt/vol), and 0.5% trypsin (wt/vol), washed, resuspended in  $\alpha$ MEM with or without Flt-Fc, and seeded onto type I collagen-coated membranes of culture inserts (Sato et al., 1998). These culture inserts were then placed in 12-well plates containing  $\alpha$ MEM supplemented with 0.1% albumin, with or without VEGF as indicated. After an overnight culture, the cells were stained for TRAP. Invasion was determined by counting before and after removal of the cells from the upper surface of the membranes, thereby rendering visible only osteoclasts or osteoclast extensions that had reached the lower surface of the membrane (Sato et al., 1998). Invasion scores were determined by taking the ratio of these two numbers. The scores of the different groups were compared with the analysis of the variance with Scheffé's *F* test.

### Cartilage Degradation

6-mm cartilage discs were punched out of 10-d-old rabbit ears, devitalized, and further processed as reported (Hauser and Vaes, 1978). They were

then placed in 96-well plates in 200  $\mu$ l  $\alpha$ MEM containing up to 50 nM MMP-13 and/or MMP-9 activated by 4-aminophenylmercuric acetate. The molar concentrations of active MMP-13 and MMP-9 were determined by active site titration with the tight-binding MMP inhibitor BB94 (Knight, 1995). The discs were incubated at 37°C under 5% CO<sub>2</sub> for up to 24 h. Hydroxyproline was measured in the media after hydrolysis in saturated Ba(OH)<sub>2</sub> (Bergman and Loxley, 1963). Under these conditions, hydroxyproline in the media increased linearly with incubation time.

### Metatarsal Cultures with mFlt-IgG

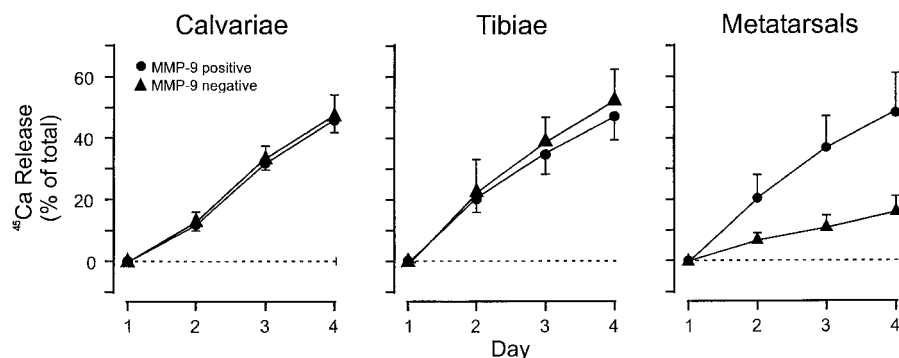
Day 17 metatarsals (day of vaginal plug is designated as day 0) from wild-type mice were dissected and cultured for 3 d on Nucleopore membrane floating on 1 ml of BGJb culture medium supplemented with 10% fetal calf serum. Control IgG or the mouse chimeric VEGF receptor mFlt-Ig was added to a final concentration of 20  $\mu$ g/ml. The medium was changed daily. After the culture, bones were fixed in 4% paraformaldehyde at 4°C overnight and processed for paraffin sections.

## Results

### Analysis of the Effect of MMP-9 Deficiency on Bone Resorption

MMPs are rate-limiting for bone resorption in cultures of embryonic day (E)18 calvariae and E17 tibiae which are rich in mature bone-resorbing osteoclasts and in cultures of E17 metatarsals that lack mature osteoclasts, but depend on recruitment of (pre)osteoclasts for resorption (Delaissé et al., 1985; Hill et al., 1994; Blavier and Delaissé, 1995; Everts et al., 1998). Osteoclasts and osteoclast precursors express MMP-9 at a high level (Reponen et al., 1994; Tezuka et al., 1994; Blavier and Delaissé, 1995; Okada et al., 1995). Therefore, we used MMP-9<sup>-/-</sup> mice to investigate the effect of MMP-9 deficiency in these calvaria, tibia, and metatarsal bone resorption models (Fig. 1). The absence of MMP-9 did not affect the demineralization rates of the calvariae and tibiae, but did reduce demineralization of metatarsals to 30% of the values found in the MMP-9-positive controls. These data suggest that MMP-9 deficiency affects resorption of calcified skeletal elements only in situations where osteoclast recruitment is a prerequisite for resorption.

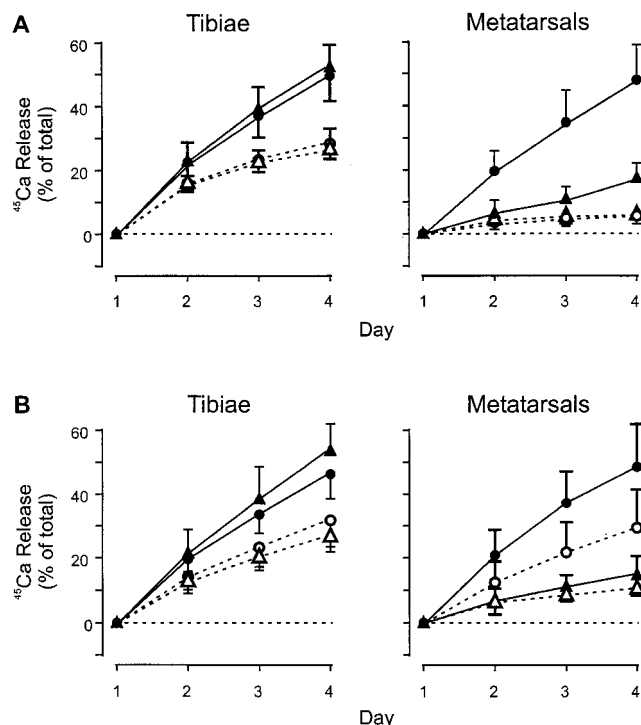
We compared the sensitivity of resorption of MMP-9-positive and MMP-9-deficient bones to various inhibitors of proteinases reported to be rate-limiting for resorption (Fig. 2). E-64, a cysteine proteinase inhibitor, reduced resorption of tibiae to similar levels in MMP-9-positive and MMP-9-deficient bones. These results suggest that the absence of effect of MMP-9 deficiency on resorption is not due to compensation by cysteine proteinases. A general MMP inhibitor produced 50% inhibition in the tibiae, and close to 100% in the metatarsal cultures, in accordance with our previous report that MMP activity is involved in resorption but is more critical for osteoclast recruitment (Blavier and Delaissé, 1995). These levels of inhibition were not influenced by MMP-9 deficiency. MMP-9 deficiency did not affect either the levels of other MMPs in extracts of freshly isolated or cultured calvariae, tibiae, and metatarsals or in their conditioned medium, as far as can be detected by gelatin/casein zymography (not shown). We interpret these data to indicate that MMPs other than MMP-9 are involved in resorption of calcified tissue itself and suggest that, instead, MMP-9 may play a role in osteoclast recruitment.



**Figure 1.** Effect of MMP-9 deficiency on the resorption of different types of bones. Calvariae of E18 embryos and tibiae and metatarsals of E17 embryos were obtained from two and eight litters, respectively, of heterozygote parents. The embryos were phenotyped as MMP-9-positive (circles) or MMP-9-negative (triangles). The bone explants were cultured for the indicated times. Each point shows the mean  $\pm$  SD of  $^{45}\text{Ca}$  release determined in independent cultures of 41 MMP-9-positive and 23 MMP-9-negative metatarsals or tibiae, or 9 MMP-9-positive and 4 MMP-9-negative calvariae. Significant differences in  $^{45}\text{Ca}$  release from MMP-9-positive and MMP-9-negative explants were seen only for the metatarsals ( $P < 0.05$ ).

### Parallel Changes in MMP-9 and Osteoclast Activities at Early Stages of Bone Development

We next evaluated the expression of MMP-9 at critical steps of osteoclast recruitment. We analyzed metatarsals of



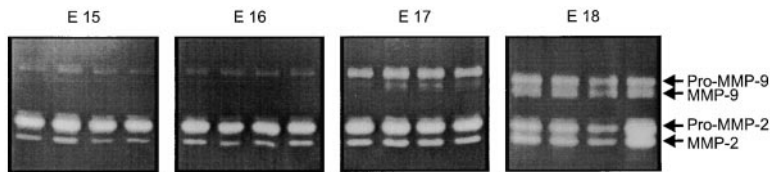
**Figure 2.** Effect of proteinase inhibitors on the resorption of metatarsals and tibiae. Tibiae and metatarsals of E17 embryos were obtained from three (A) or five (B) litters of heterozygote parents. The embryos were phenotyped as MMP-9-positive (circles) or MMP-9-negative (triangles). The bone explants were cultured in the absence (filled symbols) or presence (corresponding open symbols) of 2.5  $\mu\text{M}$  BB94 (A) or of 48  $\mu\text{M}$  E64 (B). Each point shows the mean  $\pm$  SD of  $^{45}\text{Ca}$  release determined in 14 independent cultures of MMP-9-positive and 12 independent cultures of MMP-9-negative explants, each with and without BB94 (A), or in 29 independent cultures of MMP-9-positive and 12 independent cultures of MMP-9-negative explants, each with and without E-64 (B). All the inhibitors significantly inhibited  $^{45}\text{Ca}$  release, with the exception of E64 on MMP-9-negative metatarsals after 2 d of culture ( $P < 0.05$ ).

mouse embryos at three well characterized developmental stages (Scheven et al., 1986): (a) at E15 and E16, when osteoclast precursors and endothelial cells are in the mesenchyme surrounding the bone rudiments well before the presence of a marrow cavity and of mature osteoclasts; (b) at E17, when the osteoclast precursors become TRAP<sup>+</sup>; and (c) at E18, when they invade into the bone together with endothelial cells and differentiate into mature osteoclasts that resorb the calcified cartilage of the core of the diaphysis. When we analyzed extracts of these metatarsals at these different developmental stages by zymography (Fig. 3), we detected MMP-9 zymogen as a faint band at E15 and E16, and as a strong band at E17. At E18 we detected two bands corresponding to zymogen and active forms. Thus the changes in MMP-9 levels and activation state occur concomitantly with invasion of TRAP<sup>+</sup> cells into the bone rudiment. MMP-2 levels changed less dramatically during this period although a higher proportion of activated MMP-2 was found with increasing development.

We then localized MMP-9 mRNA and protein in these metatarsals, and compared their localization to (pre)osteoclasts and endothelial cells, the cell types showing invasive activity. We identified these cells by staining them for TRAP and CD34, respectively. We prepared sets of adjacent sections stained for TRAP/CD34, and MMP-9 mRNA or MMP-9 immunoreactivity (Fig. 4). We detected MMP-9 at E17, the developmental stage at which the osteoclast precursors become TRAP<sup>+</sup> (Scheven et al., 1986). The analysis of the adjacent sections showed that the strong MMP-9 mRNA and protein signals were restricted to TRAP<sup>+</sup> cells, and did not correspond to endothelial cells. Therefore, the major source of MMP-9 in primitive metatarsals that are about to undergo invasion at E17 is the (pre)osteoclasts. These observations show that there is a close spatial-temporal correlation between MMP-9 and invasion of the osteoclasts into the core of the developing bones.

### Histomorphometric Analysis of the Effect of MMP-9 Deficiency on Osteoclast Recruitment

To investigate whether MMP-9 is actually required in this invasion process, and to characterize its role in osteoclast recruitment by direct observations, we analyzed the effect of MMP-9 deficiency on the numbers and distribution of



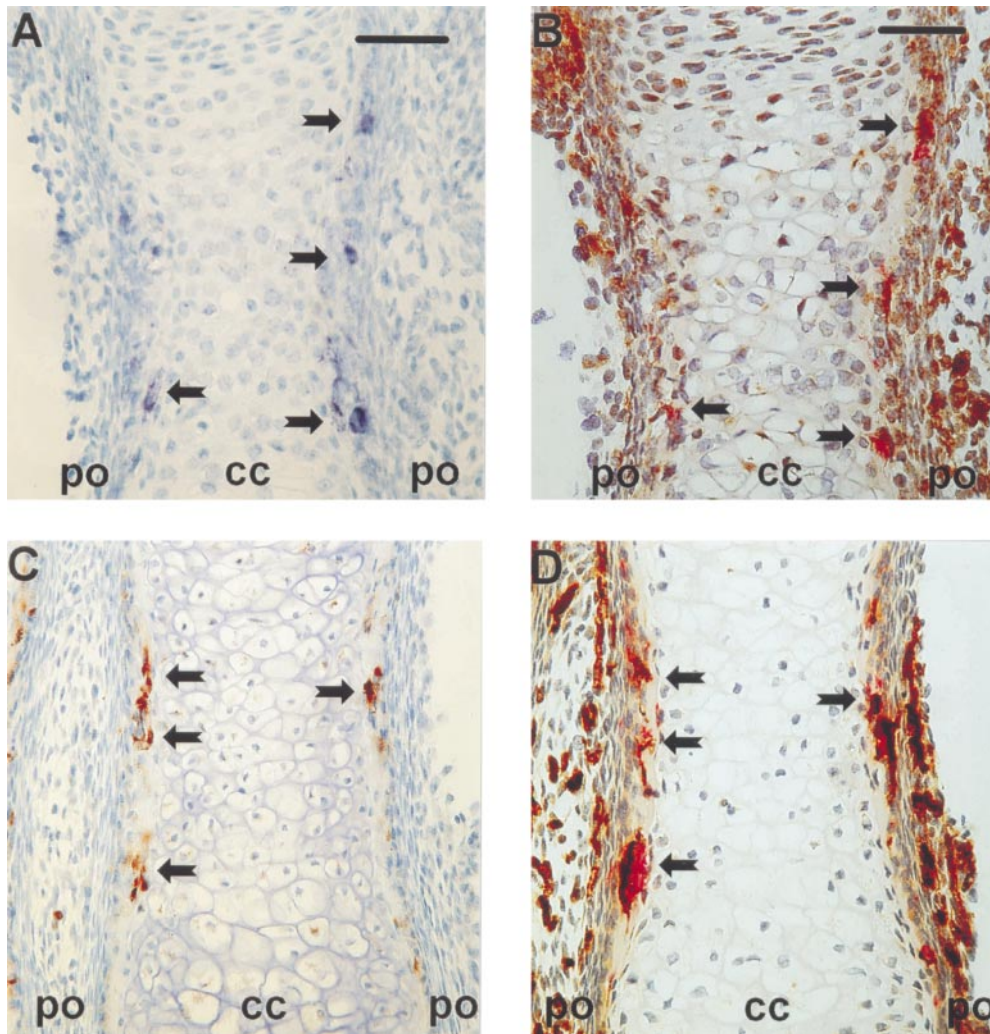
**Figure 3.** Gelatin zymography of extracts of metatarsals at different developmental stages. Metatarsal triads were isolated from four mouse embryos at E15, E16, E17, and E18. Extracts were obtained and analyzed by zymography as explained in Materials and Methods. Each lane is representative of one embryo. Pro-MMP-9 and Pro-MMP-2 are the zymogens of MMP-9 and MMP-2, respectively.

TRAP<sup>+</sup> cells and their nuclei in the metatarsals of E17, E18, and E19 embryos (Fig. 5). At E17, there were only a few TRAP<sup>+</sup> cells in the mesenchyme surrounding the bone, and none of them had invaded the calcified cartilage in agreement with earlier reports on the wild-type mice (Blavier and Delaissé, 1995). We did not detect differences between MMP-9-positive and MMP-9-negative bones with respect to number, size, and distribution of TRAP<sup>+</sup> cells. Thus, there is no indication that MMP-9 has an obligatory role in the differentiation of preosteoclasts into TRAP<sup>+</sup> cells before bone invasion.

At E18 and E19, MMP-9-positive bones (+/- from MMP-9<sup>+/-</sup> × MMP-9<sup>-/-</sup> matings) showed the typical features of recruitment of TRAP<sup>+</sup> cells (Blavier and Delaissé, 1995). Thus, the total numbers of nuclei increased up to three and four times the values found at E17, in

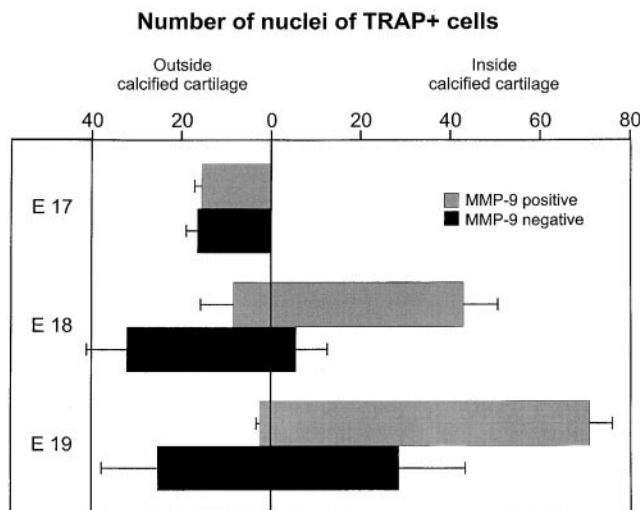
agreement with the concept that there is a continuous differentiation of TRAP<sup>-</sup> precursors into TRAP<sup>+</sup> cells. At the same time there was a dramatic shift in the distribution of TRAP<sup>+</sup> cells from the surrounding mesenchyme into the calcified core of the diaphysis. From E17 to E18 and E19, the proportion of nuclei in the core of the diaphysis increased from 0 to 81% and 98%, respectively. During this invasion, the TRAP<sup>+</sup> cells fused progressively into multinucleated osteoclasts that resorbed the septae of the calcified cartilage, and transformed the core of the bone into a "marrow" cavity.

MMP-9<sup>-/-</sup> bones from embryos of the same litter and of the same age differed from these MMP-9<sup>+/-</sup> bones mainly in two respects. First, the increase in the number of nuclei of TRAP<sup>+</sup> cells was only 70% of that of MMP-9<sup>+/-</sup> bones (Fig. 5). The average number of nuclei per osteoclast, how-



**Figure 4.** In situ hybridization and immunostaining of MMP-9 in sections of diaphysis of developing metatarsals. Two sets of adjacent sections of E17 metatarsals were prepared (A, B and C, D). In each set, one section was hybridized with antisense MMP-9 probe (A), or immunostained for MMP-9 (C); their respective adjacent sections were stained both for CD34 immunoreactivity and TRAP activity (B and D). Strong hybridization signals (A) and MMP-9 immunoreactivity (C) are seen outside the calcified cartilage (cc) (arrows). Signals for TRAP activity (red) in the adjacent sections (B and D) (arrows) correspond to the localizations of the MMP-9 hybridization (A) and immunoreactivity (C) signals, respectively. CD34 signals (brown) (B and D) are abundant in the periosteal cell layer (po) and do not have clearly matching MMP-9 hybridization (A) or immunoreactivity signals (C). Bars, 50  $\mu$ m.



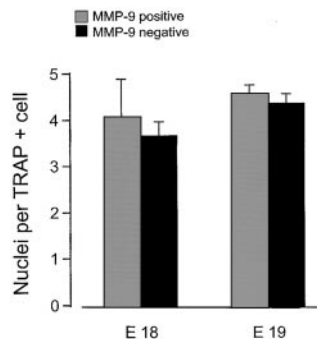


**Figure 5.** Effect of MMP-9 deficiency on number and distribution of TRAP<sup>+</sup> cells in developing metatarsals. Litters of E17, E18, and E19 embryos were obtained from MMP-9<sup>+/-</sup> and MMP-9<sup>-/-</sup> parents. The embryos were genotyped as MMP-9<sup>+/-</sup> or MMP-9<sup>-/-</sup>. Metatarsal triads were analyzed with respect to number of nuclei of TRAP<sup>+</sup> cells localized outside or inside the calcified cartilage. Means  $\pm$  SD were calculated for each age group and genotype. Total numbers of embryos were as follows: at E17, 12 MMP-9<sup>+/-</sup> and 11 MMP-9<sup>-/-</sup> (pool of 3 litters); at E18, 31 MMP-9<sup>+/-</sup> and 20 MMP-9<sup>-/-</sup> (pool of 7 litters); and at E19, 9 MMP-9<sup>+/-</sup> and 8 MMP-9<sup>-/-</sup> (pool of 3 litters). Mean counts outside the calcified cartilage are shown by a block to the left of the zero axis, and mean counts inside the calcified cartilage by a block to the right. Each horizontal bar resulting from the alignment of these two blocks represents the mean number of total nuclei in one section of a metatarsal triad. At E18 and E19, MMP-9<sup>-/-</sup> and MMP-9<sup>+/-</sup> metatarsals differed significantly with respect to both total number of nuclei of TRAP<sup>+</sup> cells and their proportion in the calcified cartilage ( $P < 0.05$ ). In contrast, at E17 no significant differences were found.

ever, was not affected (Fig. 6). This observation suggests that the lack of MMP-9 does not affect cell fusion. Second, and more importantly, MMP-9 deficiency resulted in a delayed invasion of TRAP<sup>+</sup> cells into the calcified cartilage (Figs. 5 and 7). At E18 and E19, the proportions of nuclei in the calcified cartilage were 20 and 40%, respectively, of those in MMP-9-positive bones. The osteoclasts of the MMP-9-negative bones tended then to accumulate at the interface between the osteoid (i.e., unmineralized collagen) of the bone collar and the calcified cartilage. During this period, the distribution of endothelial cells inside and outside the calcified cartilage was closely coordinated with that of TRAP<sup>+</sup> cells, whether MMP-9 was lacking or not. Endothelial cells appeared inside the calcified cartilage only when TRAP<sup>+</sup> cells had invaded the calcified cartilage (Fig. 7). Thus, the main result of MMP-9 deficiency was an impaired migration of TRAP<sup>+</sup> cells and endothelial cells from the periosteum to the calcified cartilage.

#### Effect of MMP-9 Deficiency on Invasion of Type I Collagen by Osteoclasts

When the TRAP<sup>+</sup> cells migrate from the periosteum to the calcified cartilage, they must overcome a barrier of unmineralized bone matrix, called osteoid, that comprises



**Figure 6.** Effect of MMP-9 deficiency on number of nuclei per TRAP<sup>+</sup> cell. The number of nuclei per TRAP<sup>+</sup> cell were calculated from the counts of cells and nuclei shown in Fig. 5, and are shown as mean  $\pm$  SD.

mainly type I collagen (Blavier and Delaissé, 1995). Migration of osteoclasts through type I collagen is inhibited by a general MMP inhibitor (Sato et al., 1998). To evaluate whether MMP-9 is critical for migration of osteoclasts through collagen, we compared migration rates of MMP-9-positive and MMP-9-negative osteoclasts through collagen-coated membranes. A general MMP inhibitor strongly blocked invasion of both phenotypes to the same extent. However, MMP-9<sup>-/-</sup> osteoclasts were not significantly less invasive (Fig. 8). These data indicate that a metalloproteinase (or metalloproteinases) different from MMP-9 is critical for the invasive activity of osteoclasts into collagen.

#### Effect of MMP-9 on Solubilization of Cartilage

To determine whether the delayed invasion into cartilage relates to a role of MMP-9 in cartilage solubilization, we investigated the effect of MMP-9 on cartilage degradation in the presence and absence of MMP-13 (collagenase-3), an MMP that is highly expressed by the hypertrophic chondrocytes at this developmental stage (Blavier and Delaissé, 1995) and strongly binds to collagen (Gillet et al., 1977; Delaissé et al., 1993). We found that MMP-9 rendered the collagenolytic activity of MMP-13 twice as efficient, but had almost no collagenolytic activity by itself (Table I). This collagenolysis-enhancing effect of MMP-9 may favor the penetration of the cells into the cartilage core of the diaphysis.

#### Role of VEGF in the Recruitment of Osteoclasts

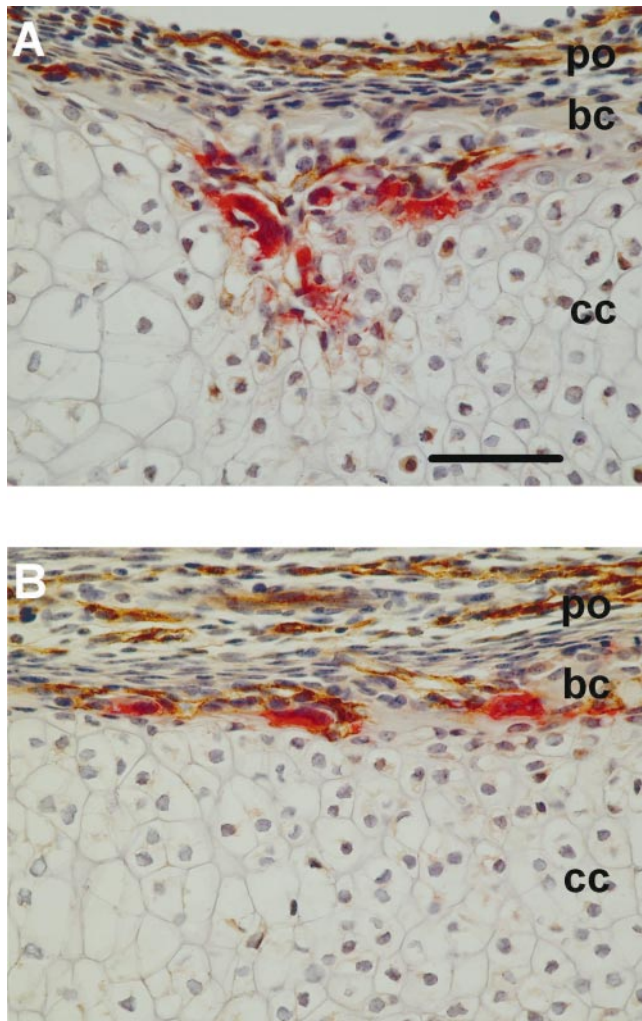
At the growth plate, hypertrophic cartilage expresses VEGF, and inhibition of VEGF activity blocks the recruit-

**Table I.** Effect of MMP-9 on Solubilization of Type II Collagen from Cartilage

| Additive                | Collagen solubilized<br>%* |
|-------------------------|----------------------------|
| MMP-9                   | 19 $\pm$ 4                 |
| MMP-13                  | 100                        |
| MMP-9 + MMP-13          | 219 $\pm$ 7                |
| MMP-9 + MMP-13 + GM6001 | 6 $\pm$ 2                  |

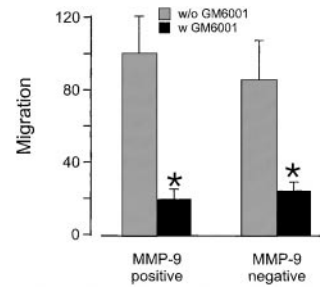
Cartilage discs were incubated for 12 or 24 h in the presence of 50 nM of the indicated MMP. The concentration of MMP inhibitor GM6001 was 10  $\mu$ M. Collagen solubilizations were normalized to the levels obtained in the presence of MMP-13 (average of 19.7 nmol hydroxyproline per 24 h), and are shown as means  $\pm$  SD of three independent incubations.

\*Percentage of solubilization by MMP-13.



**Figure 7.** Sections through the diaphysis of MMP-9-positive and MMP-9-negative metatarsals showing invasion and lack of invasion, respectively, by (pre)osteoclasts and endothelial cells at E18. Sections of MMP-9-positive (A) and MMP-9-negative (B) metatarsals of E18 embryos were obtained as explained in Fig. 5 and stained for TRAP activity and CD34 immunoreactivity to visualize (pre)osteoclasts (red) and endothelial cells (brown), respectively. MMP-9-positive metatarsals show large mature osteoclasts and endothelial cells that have invaded calcified cartilage (cc). MMP-9-negative metatarsals do not show osteoclasts or endothelial cells in calcified cartilage, but show well (pre)osteoclasts in the bone collar (bc). Endothelial cells are also abundant in the perosteal cell layer (po) in both phenotypes. Bar, 50  $\mu$ m.

ment of MMP-9-positive and TRAP<sup>+</sup> cells (Gerber et al., 1999). We found that hypertrophic cartilage of E17 metatarsals, before osteoclast recruitment, also expressed VEGF. There were no differences in VEGF expression in MMP9<sup>+/+</sup> or MMP-9<sup>-/-</sup> metatarsals (Fig. 9 A). To determine the role of VEGF in the recruitment of osteoclasts into the hypertrophic cartilage, we blocked VEGF activity in cultures of E17 metatarsals. Metatarsals cultured for 3 d in the presence of a soluble VEGF receptor, mFlt-IgG, showed very few TRAP<sup>+</sup> cells inside the hypertrophic cartilage. This inhibition was not as complete as that produced by a broad spectrum MMP inhibitor, as was also seen for MMP-9<sup>-/-</sup> metatarsals. In contrast, those metatarsals cul-



**Figure 8.** Comparative effect of MMP-9 deficiency and of a general MMP inhibitor on osteoclast migration through type I collagen. Osteoclasts of MMP-9-positive and MMP-9-negative mice were cultured on collagen-coated filters in the presence and absence of 10  $\mu$ M GM6001 (average of 227 osteoclasts/filter).

Their invasion through the collagen was evaluated as explained in Materials and Methods, and is shown as means  $\pm$  SD of three cultures. \*Significant effect compared with osteoclasts of MMP-9-positive mice cultured without inhibitor ( $P < 0.05$ ).

tured in the presence of a control IgG of the same isotype showed extensive invasion of TRAP<sup>+</sup> cells into hypertrophic cartilage (Fig. 9 B). These data show that VEGF is involved in (pre)osteoclast recruitment in the core of the diaphysis, similar to that in the growth plates.

To further explore whether VEGF acts directly on osteoclasts and exerts chemotactic activity, we seeded osteoclasts differentiated from bone marrow precursors in vitro in the upper chamber of culture inserts with collagen-coated membranes, and determined their migration to the lower surface of the membranes in the presence of increasing concentrations of VEGF in the lower chamber. VEGF stimulated migration of the cells (Fig. 10). This effect of VEGF was abolished by addition of hFlt-Fc to the upper chamber. These observations show that VEGF can act directly on osteoclasts and is chemotactic for them.

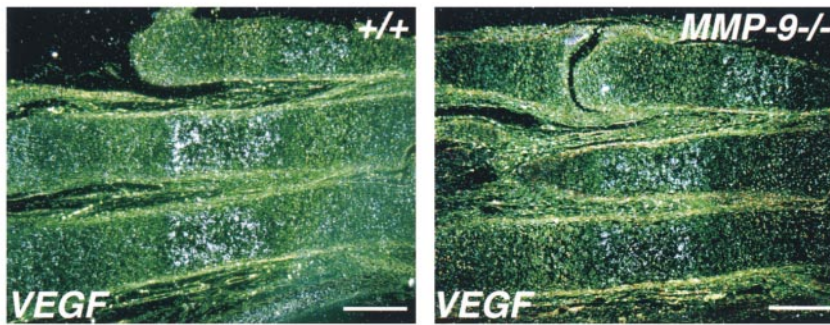
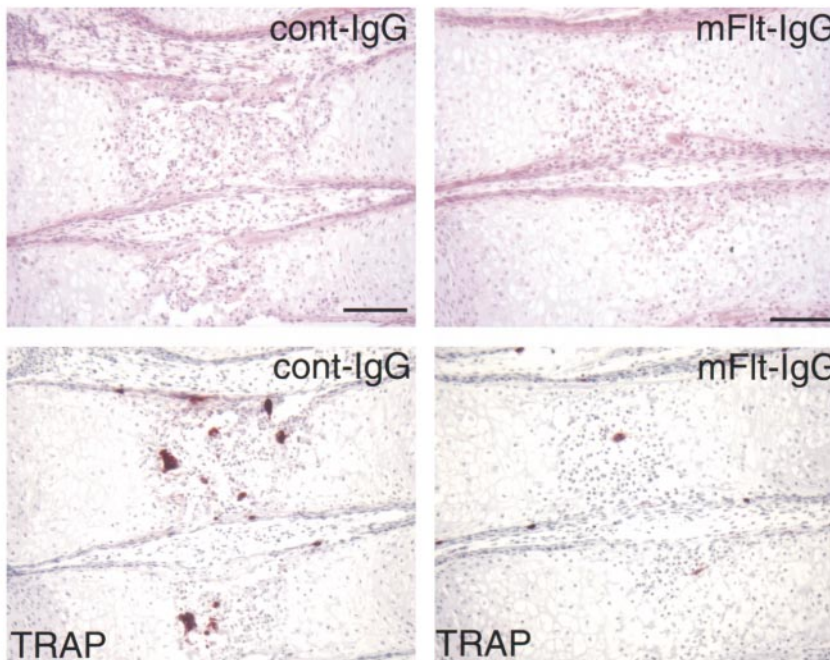
## Discussion

The development of the skeleton requires targeting of the destructive activity of the osteoclast to specific sites of the bone. Proteinases and local factors play an important role in regulating this process, but their identity and their mode of action have not been determined. Our work shows that MMP-9 from osteoclast and/or osteoclast-like cells plays an important role in inducing and expanding the primitive marrow cavity of long bones. This role is a permissive function to facilitate osteoclast invasion into the cartilaginous core of the bone rudiments, rather than an involvement in removal of mineralized matrix. Other MMPs are, however, critical at other stages of the recruitment process. Also, we show that VEGF is among the rate-limiting factors inducing the formation of the primitive marrow cavity and is a chemoattractant for osteoclasts. This indicates that endothelial cells are not the only cell type mediating VEGF-induced cartilage invasion. Since MMP-9 is able to release ECM-bound VEGF (Bergers et al., 2000), our findings suggest an integrated action of MMP-9 and VEGF in skeletal development.

### MMP-9 Is Not Involved in Solubilization of Mineralized Matrix by Osteoclasts

Osteoclasts that resorb mineralized matrix typically are polarized cells, forming a sealed-off resorption compartment upon attachment onto the surface of the mineralized matrix. Inhibitors of the cysteine proteinase cathepsin K



**A****B**

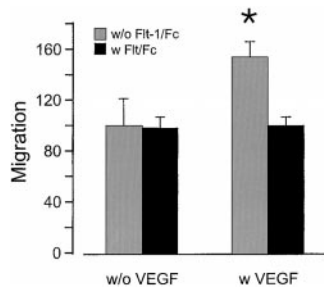
**Figure 9.** Effect of blocking VEGF activity on recruitment of TRAP<sup>+</sup> cells into developing metatarsals. (A) Dark field photographs of sections of E17 wild-type (+/+) and MMP-9 null (MMP-9<sup>-/-</sup>) metatarsals hybridized with a <sup>35</sup>S-labeled VEGF antisense probe. (B) Adjacent sections of metatarsals cultured for 3 d in control IgG or mFlt-IgG stained with hematoxylin and eosin (upper panels) or for TRAP activity (lower panels). Bars, (A) 200  $\mu$ m; (B) 320  $\mu$ m.

and general MMP inhibitors inhibit collagen degradation in this zone (Everts et al., 1998). The effect of MMP inhibitors coupled with the high expression of MMP-9 in osteoclasts led to the hypothesis that MMP-9 is the critical MMP for bone matrix solubilization (Tezuka et al., 1994; Okada et al., 1995). However, bone histology of the MMP-9 knockout mice does not reveal a lack of osteoclastic resorption of mineralized matrix (Vu et al., 1998). In further support of the lack of a role of MMP-9 in solubilization of mineralized matrix, we show that specialized bone resorption models, which respond to general MMP inhibitors (Delaissé et al., 1985; Hill et al., 1994; Blavier and Delaissé, 1995) (see Fig. 2), have the same bone degradation rates in MMP-9-negative and MMP-9-positive bones, even when bone resorption was stimulated with bone resorbing agents like parathyroid hormone and 1,25(OH)<sub>2</sub>D<sub>3</sub>. In calvariae, where MMPs have proved to be particularly important for matrix solubilization (Everts et al., 1999), MMP-9 deficiency was without effect. Cathepsin K, which is very efficient for matrix solubilization by osteoclasts (Saftig et al., 1998), did not compensate for MMP-9 because a cathepsin K inhibitor reduced resorption of tibiae to similar levels in MMP-9-negative and MMP-9-positive

bones. Thus, the identity of the critical MMP involved in matrix solubilization in the resorption zone remains to be determined.

***MMP-9 Is a Major MMP for Osteoclast Recruitment in Primitive Long Bones, and Is Specifically Required for the Passage of the Cells through Their Osteoid–Cartilage Interface***

The first destructive task of osteoclasts in primitive long bones is the formation of the marrow cavity. Preosteoclasts are recruited into the bone from the surrounding mesenchyme, a process that is completely inhibited by general MMP inhibitors (Blavier and Delaissé, 1995). We have now identified MMP-9 as a major MMP for this recruitment process. This role of MMP-9 is also supported by observations in bones of wild-type mice, since there is an excellent correlation between MMP-9 and invasion of osteoclasts into the core of the diaphysis. However, MMPs distinct from MMP-9 also participate in this recruitment, since the inhibition induced by the absence of MMP-9 is only partial whereas the inhibition generated by general MMP inhibitors is complete.



**Figure 10.** Effect of VEGF on osteoclast migration. Osteoclasts were cultured overnight on collagen-coated membranes of culture inserts (average of 1,032 osteoclasts/insert). These inserts were placed in 12-well plates containing the indicated concentration of VEGF. 100 ng/ml Flt-1/Fc was added to the

culture inserts where indicated. The migrations were scored as explained in Materials and Methods, and are shown as mean  $\pm$  SD of four cultures. \*Significant effect compared with osteoclasts cultured without additive ( $P < 0.05$ ).

The biological events required by this recruitment are the generation of preosteoclasts in the mesenchyme surrounding the bone, their maturation, and their migration into the core of the diaphysis. It is clear that the main role of MMP-9 (and/or other MMPs) relates to the latter migration because lack of MMP-9 (or of MMP activity) leads to an accumulation of TRAP<sup>+</sup> cells around the diaphysis, and does not prevent the formation of multinucleated cells. The generation of TRAP<sup>+</sup> cells in E18 and E19 in the absence of MMPs is somewhat smaller (Blavier and Delaissé, 1995; this work). This may be due to the modulating effect that resorption may exert on the recruitment of new osteoclasts (Van De Wijngaert et al., 1988), or to decreased VEGF bioavailability which may participate in osteoclast differentiation (Niida et al., 1999).

There are several critical steps along the migration route of the TRAP<sup>+</sup> cells. The preosteoclasts must leave the periosteal cell layer, move through a 12–25- $\mu$ m seam of osteoid whose inner edge is slightly and discontinuously mineralized, and finally invade the outer cartilage septae that are also discontinuously mineralized (Blavier and Delaissé, 1995). In the absence of MMP-9, the TRAP<sup>+</sup> cells tend to accumulate at the osteoid–cartilage interface, suggesting that their movement through osteoid is not affected. Experiments with type I collagen gels in invasion chambers confirmed that the intrinsic ability of osteoclasts to move through collagen is not significantly affected by the absence of MMP-9. This is in contrast to the effects of general MMP inhibitors, which block the migration of the (pre)osteoclasts through osteoid in E17 metatarsals (Blavier and Delaissé, 1995) or through type I collagen in invasion chambers (Sato et al., 1998; this study). This shows that other (or another) MMPs are especially important for the migration through osteoid, which involves only focal lysis (Blavier and Delaissé, 1995). A good candidate is MT1-MMP, a membrane-bound collagenolytic proteinase that has been identified in invadopodia and at the leading edge of moving osteoclasts (Sato et al., 1997). MT1-MMP null mice show bone and cartilage defects that have not been investigated in detail (Holmbeck et al., 1999).

### **What Is the Molecular Mode of Action of MMP-9 for Cartilage Invasion?**

Our data are compatible with several molecular modes of action of MMP-9 in cartilage invasion. First, MMP-9 may speed up cartilage solubilization. This is interesting because such proteolytic action is required exactly at the

point of the migration route where MMP-9 becomes necessary. At E17 this cartilage matrix is discontinuously mineralized (Blavier and Delaissé, 1995). The classical resorptive machinery of the osteoclast is responsible for solubilizing the mineralized cartilage, as shown by the effects of classical inhibitors of their resorptive activity, including bisphosphonates and cysteine proteinase inhibitors (Blavier and Delaissé, 1995). In contrast, these inhibitors do not inhibit invasion of the osteoclasts into the core of the diaphysis, and this invasion largely proceeds by passage through nonmineralized septae (Blavier and Delaissé, 1995). Since this passage requires MMP-9, it appears that the role of MMP-9 relates to solubilization of nonmineralized septae. This is reminiscent of cartilage invasion at the growth plate: the transversal septae are nonmineralized and their invasion does not require resorptive activity of osteoclasts (Schenk et al., 1967), but requires MMP-9 (Vu et al., 1998). MMP-9 is present at the edge of the last transversal septa and in debris of type II collagen at various states of digestion (Vu et al., 1998; Lee et al., 1999). Our test tube assays show that MMP-9 stimulates unmineralized cartilage degradation in the presence of MMP-13. Interestingly, MMP-13 is highly expressed in the hypertrophic chondrocytes filling the diaphysis of embryonic metatarsals, as well as in the growth plates (Blavier and Delaissé, 1995; Gack et al., 1995), and binds cartilage septae (Lee et al., 1999). It has been proposed that MMP-9 and MMP-13 cooperate for degrading the unmineralized cartilage of these septae (Lee et al., 1999). Our experiments provide direct support to this hypothesis. One may speculate that MMP-9 speeds up the degradation of proteoglycans, thereby rendering the collagen more accessible for degradation by MMP-13. Alternatively, MMP-9 may promote the digestion of the collagen that was destabilized by the attack of MMP-13. However, it is unclear to what extent the effect of MMP-9 on collagenolysis is essential for cartilage invasion, since cartilage invasion at the secondary site of ossification is only minimally affected by the lack of MMP-9 (Vu et al., 1998), although this invasion also requires breaching of cartilage septae. Both MMP-9 and MMP-13 are normally expressed at these sites (Delaisé, unpublished results).

Second, MMP-9 may favor cartilage invasion by releasing growth and differentiation factors. Proteolytic release of matrix-bound components from E17 metatarsals and growth plates appears to be involved in recruitment of TRAP<sup>+</sup> cells and endothelial cells, respectively (Van De Wijngaert et al., 1988; Vu et al., 1998). Among the factors affecting osteoclast recruitment in both growth plate and E17 metatarsals are M-CSF (Felix et al., 1990; Hofstetter et al., 1995; Jemtland et al., 1998), TGF- $\beta$  (Dieudonné et al., 1991), and VEGF (Gerber et al., 1999). That active TGF- $\beta$  and VEGF are made bioavailable by MMP-9 (Bergers et al., 2000; Yu and Stamenkovic, 2000) may be particularly relevant to the recruitment process. VEGF function is required in growth plate invasion (Gerber et al., 1999) and diaphysis invasion (this work); however, it is absent at the secondary site of ossification of the epiphysis (Werb, unpublished results) where the role of MMP-9 is also less important (Vu et al., 1998). A hypothesis that unifies these observations is that the major mode of action of MMP-9 is to make VEGF functional as a chemoattractant and promote invasion.

Still other modes of action of MMP-9 in diaphysis invasion are possible. Several molecules appear to be positioned at the osteoid–cartilage interface, i.e., precisely at the site where MMP-9 becomes important. These include molecules with strong effects on cell motility, such as transferrin, bone sialoprotein, osteopontin, and osteonectin (Gentili et al., 1994; Sommer et al., 1996). Interestingly, MMP-9 cleaves osteopontin and osteonectin molecules in their functional domain (Sasaki et al., 1997; Delaissé, unpublished results). The adhesive properties of osteopontin are strongly affected by this fragmentation (Senger et al., 1994; Xuan et al., 1994). Finally, osteoclasts express CD44 (Nakamura et al., 1995), an adhesion molecule with a strong affinity for hyaluronic acid that is abundant in cartilage. Hyaluronic acid may induce clustering of CD44 and MMP-9 on their plasma membrane, thereby promoting invasiveness, as reported for cancer cells (Yu and Stamenkovic, 1999).

### **Relative Importance of Osteoclasts, Chondroclasts, and Endothelial Cells in Cartilage Invasion**

Several cell types that are able to express MMP-9 are present in the area of the invasion front of the growth plate. They include endothelial cells, chondroclasts, and osteoclasts. Chondroclasts refer to the cells against the nonmineralized transversal septae, whereas osteoclasts refer to cells along the mineralized longitudinal trabeculae (Vu et al., 1998). These two cell types show inverse amounts of MMP-9 and TRAP at early developmental stages, chondroclasts being richer in MMP-9 and poorer in TRAP (Jemtland et al., 1998). Moreover, chondroclasts do not form a ruffled border to the same extent as typically polarized, resorbing osteoclasts (Nordahl et al., 1998). We did not detect distinct osteoclast and chondroclast populations in diaphysis invasion. This may be due to the random distribution of mineralized and nonmineralized portions of cartilage in the primitive diaphysis (Blavier and Delaissé, 1995), in contrast to the situation in the growth plate (Schenk et al., 1967). At least some chondroclasts may derive from a cell lineage other than osteoclasts, because MMP-9–positive cells are present and growth ossification takes place in osteopetrotic mice (Fos<sup>-/-</sup>; Grigoriadis et al., 1994). However, osteoclasts can exhibit distinct phenotypes depending on their microenvironment, such as mineral (Gravallese et al., 1998), and distinct osteoclast activities do require functional and structural reorganizations, typical of epithelial–mesenchymal transitions (Nakamura et al., 1996). In particular, the polarized resorbing osteoclast secretes the cysteine proteinase cathepsin K into the acidic resorption zone to solubilize the mineralized matrix, whereas the rounded, nonpolarized osteoclasts depend on MMPs and not on cysteine proteinases for migration in nonmineralized collagen (Sato et al., 1998). The nonpolarized osteoclast-related cell that invades nonmineralized cartilage in the growth plate and diaphysis depends on MMP-9 for this invasion (Vu et al., 1998; this work).

Another important question is the identity of the target cells of the VEGF. VEGF effects have often been interpreted in the context of chemotaxis on endothelial cells. However, osteoclasts express VEGF receptor 1 (Flt-1), and VEGF can support osteoclast generation (Niida et al., 1999). Our work also shows that VEGF acts directly on os-

teoclasts and promotes their invasive activity. The fact that both endothelial cells and osteoclasts are responsive to VEGF fits well the observation that invasion of the diaphysis of the primitive metatarsals is approximately simultaneous for both cell types. Through these mechanisms VEGF serves to coordinate ECM degradation and recruitment of endothelial cells and osteoclasts.

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